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Molecular mechanism of agonist recognition by the ligand-binding core of the ionotropic glutamate receptor 4

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Abstract The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) class of ionotropic glutamate receptors comprises four different subunits: iGluR1/iGluR2 and iGluR3/iGluR4 forming two subgroups. Three-dimensional structures have been reported only of the ligand-binding core of iGluR2. Here, we present two X-ray structures of a soluble construct of the R/G unedited flip splice variant of the ligand-binding core of iGluR4 ($iGluR4_i$(R)-S1S2) in complex with glutamate or AMPA. Subtle, but important differences are found in the ligand-binding cavity between the two AMPA receptor subgroups at position 724 (Tyr in iGluR1/iGluR2 and Phe in iGluR3/iGluR4), which in iGluR4 may lead to displacement of a water molecule and hence points to the possibility to make subgroup specific ligands.

Keywords: Ionotropic glutamate receptor; IGluR4; AMPA; Glutamate; Flip; Crystal structure

1. Introduction

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors belong to the family of ionotropic glutamate receptors (iGluRs) and are the primary mediators of fast synaptic transmission in the central nervous system [1] and show a broad distribution. These receptors engage in regulation of synaptic strength, a mechanism putatively underlying learning and memory [2]. However, AMPA receptors are also implicated in a number of neurological diseases [3].

AMPA receptors are composed of four types of subunits, designated iGluR1-iGluR4. Most, if not all, AMPA receptors form heterotetramers, consisting of symmetric dimer-of-dimers of iGluR2 and either iGluR1, iGluR3 or iGluR4, and the stoichiometry of subunits has been shown to determine channel function and receptor trafficking [4]. The ligand-binding core is present in the extracellular part of the receptors and consists of two domains D1 and D2. Upon binding of an agonist, these two domains move towards each other, leading to opening of the ion channel pore; a so-called Venus-flytrap mechanism [5].

Alternative splicing and RNA editing within the ligand-binding core of AMPA receptors further modifies receptor function. Flip/flop alternative splicing of 38 amino acids in the S2 segment prior to the third transmembrane region creates a range of functional AMPA receptor subunits that enable modulation of the rate of desensitization and recovery from desensitization [1]. Similar effects have been demonstrated for RNA editing at the R/G site preceding the splice variant region [6,7].

To further study the functional role of different AMPA receptors, compounds that selectively target individual subunits are essential. Today, compounds are available that selectively target either iGluR1/iGluR2 or iGluR3/iGluR4 [8–12]. The structure determination of a ligand-binding core construct of iGluR2 [5] has stimulated numerous structural and functional studies on this receptor. However, until now the other three AMPA receptor subunits have not been subjected to structure determination. Here, we present two X-ray structures of the ligand-binding core of the R/G unedited form of the flip splice variant of iGluR4 ($iGluR4_i$(R)-S1S2) with bound glutamate or AMPA. These structures allow a detailed analysis of structural similarities and dissimilarities within the AMPA receptor family, which may be of importance for receptor function and ligand selectivity.

2. Materials and methods

2.1. Expression and purification of $iGluR4_i$(R)-S1S2

The S1 and S2 segments constituting the $iGluR4_i$(R)-S1S2 were polymerase chain reaction (PCR)-amplified from full-length iGluR4 using the primers: S1-forward: ctcggatcccgtggcagaacagtggttgtaaccac; S1-reverse: tatgggggtacctttttgatcatgatagagatg; S2-forward: caaaaaagtaccccatagaaagtgcagaagacc and S2-reverse: gctgtgctcgagttatccacatttcacctttatcgta. PCR-products were digested with restriction enzymes and ligated into pET-32a(+) (Novagen, WI, USA). The protein was

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Br-HIBO, (*S*)-α-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid; Cl-HIBO, (*S*)-α-amino-4-chloro-3-hydroxy-5-isoxazolepropionic acid; CPW399, (*S*)-2-amino-3-(1,3,5,6,7-pentahydro-2,4-dioxocyclopenta[*e*] pyrimidin-1-yl)propanoic acid; iGluR, ionotropic glutamate receptor; $iGluR2_i$(R)-S1S2J, ligand-binding core of the R/G unedited flip splice variant of iGluR2; $iGluR2_o$(G)-S1S2J, ligand-binding core of the R/G edited flop splice variant of iGluR2; $iGluR4_i$(R)-S1S2, ligand-binding core of the R/G unedited flip splice variant of iGluR4

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expressed in the *Escherichia coli* cell line Origami 2 (Novagen) as a thioredoxin fusion protein of ca. 47 kDa. An overnight culture was grown in HyperBroth + glucose nutrient mix (Athenaes, MD, USA) at 30 °C to an OD_{600} of ca. 2 before induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Protein was expressed overnight at 25 °C.

After cell harvesting and centrifugation, the fusion protein was purified on a HisTrap FF column (GE Healthcare Life Sciences, Hørsholm, Denmark) and eluted with 50 mM sodium phosphate pH 7.4, 0.5 M sodium chloride and 0.5 M imidazole. Two trypsin cleavage sites between thioredoxin and iGluR4$_i$(R)-S1S2 are present in the fusion protein, which upon digestion with trypsin in 50 mM sodium phosphate pH 6.5, 20 mM sodium chloride, 1 mM glutamate and 2 mM magnesium chloride at room temperature results in a protein of ca. 33 kDa and the intended iGluR4$_i$(R)-S1S2 of ca. 29 kDa. The digested 29 kDa construct contains an N-terminal glycine, the S1 segment [415-RTIV…MIKK-528], a Gly-Thr linker and the S2 segment [654-PIES…GECG-796]. Subsequently, the protein was purified on a HiTrap SP HP column (GE Healthcare Life Sciences) at 6 °C using 50 mM sodium phosphate pH 6.0, 1 M sodium chloride and 1 mM EDTA as elution buffer. A final purification step was done on a Superdex 75 HR column 10/30 (GE Healthcare Life Sciences) and the protein was eluted with 20 mM HEPES pH 7.0 and 10 mM sodium chloride.

2.2. Crystallization and data collection

iGluR4$_i$(R)-S1S2 was crystallized with either (*S*)-glutamate or (*S*)-AMPA, using the hanging drop vapour diffusion method at 6 °C and drops of 1 + 1 μl. For crystallization of iGluR4$_i$(R)-S1S2 with glutamate, the 10.5 mg/ml protein solution consisted of 20 mM HEPES pH 7.0, 10 mM sodium chloride and 1 mM glutamate and the crystallization buffer consisted of: 18% polyethylene glycol (PEG) 4000, 0.3 M ammonium sulfate and 0.1 M sodium acetate pH 5.5. For crystallization of iGluR4$_i$(R)-S1S2 with AMPA, the 8.3 mg/ml protein solution consisted of 20 mM HEPES pH 7.0, 10 mM sodium chloride and 10 mM AMPA and the crystallization buffer consisted of: 13% PEG4000, 0.4 M ammonium sulfate and 0.1 M sodium acetate pH 5.5.

Before data collections at beamline 911, MAX-Lab, Lund, Sweden, the crystals were cryo-cooled using glycerol. Two data sets were collected to 1.4 and 1.9 Å resolution of iGluR4$_i$(R)-S1S2 in complex with glutamate and AMPA, respectively (Table 1). The data were processed using MOSFLM [13] and SCALA within the CCP4 package [14].

2.3. X-ray structure determinations and analysis

Both structures were solved by molecular replacement using PHASER within CCP4. The structure of iGluR2-S1S2J in complex with glutamate (molA, pdb-code 1FTJ, poly-Ala) was used as the input structure in the case of iGluR4 with glutamate and the latter structure (molA) for iGluR4 with AMPA. Automated model building was performed with ARP/wARP implemented in CCP4 and further model building was done using program COOT [15]. For iGluR4$_i$(R)-S1S2 with glutamate, refinements were performed with the program REFMAC5 in CCP4. The structure of iGluR4$_i$(R)-S1S2 with AMPA was refined using PHENIX [16]. Both structures were validated in PROCHECK [17]. For statistics on refinement, see Table 1. Ligand-induced domain closures were calculated using DynDom [18]. The CCP4 program CONTACTS was used in the analysis of protein–ligand and protein–protein interactions. The program PyMOL [19] was used for the preparation of figures.

2.3.1. Protein data bank accession number. The atomic coordinates and structure factors of iGluR4$_i$(R)-S1S2 with bound glutamate and AMPA, respectively, have been deposited in the Protein Data Bank under the accession codes 3FAS and 3FAT.

2.4. Binding affinity

The binding affinities of (*S*)-glutamate and (*RS*)-AMPA at the iGluR4$_i$(R)-S1S2 soluble construct were determined by a radioligand binding competition assay. Purified construct (0.1–0.2 μg protein) was incubated with 4–6 nM (*RS*)-[5-methyl-^{3}H]-AMPA (40.0 Ci/mmol; Perkin–Elmer, Boston, MA) in the presence of 0.1 nM–0.10 mM competitor for 1.5–2 h on ice in 250 μl assay buffer (50 mM Tris–HCl, 100 mM KSCN, 2.5 mM $CaCl_2$, pH 7.2 at 4 °C; containing 10% glycerol (v/v)). Samples were filtered onto Millipore (Billerica, MA) 0.22 μm GSWP nitrocellulose filters, washed twice with 1.5 ml ice-cold assay buffer and radioactivity determined by scintillation counting. Data were analyzed using Grafit (Erithacus Software Ltd., Horley, UK) and fit as previously described [20] to determine Hill coefficient and K_i, where $K_i = K_d$ in the case of (*RS*)-AMPA.

3. Results

3.1. Glutamate and AMPA binding affinities for iGluR4$_i$(R)-S1S2

The binding affinities (K_i, mean ± SEM) of (*RS*)-AMPA (22.8 ± 1.5 nM, $n = 4$) and (*S*)-glutamate (171 ± 18 nM, $n = 3$) at iGluR4$_i$(R)-S1S2 (Fig. 1) were comparable to those previously reported at full-length iGluR4$_o$(R) (40 nM and 354 nM, respectively; [21]).

3.2. iGluR4$_i$(R)-S1S2 crystal structures

To investigate structural differences among AMPA receptors and differences in binding of ligands, we determined two high-resolution X-ray structures of iGluR4$_i$(R)-S1S2: one as a complex with glutamate (1.4 Å resolution) and one with AMPA (1.9 Å resolution). The structure of iGluR4$_i$(R)-S1S2 with glutamate contains two molecules (molA and molB) within the asymmetric unit of the crystal, whereas the structure with AMPA has three molecules (molA-molC) within the asymmetric unit. In both structures, all molecules are engaged in dimer formation (Fig. 2A), and the symmetrical dimer is similar to

Table 1
Crystallographic and structural information on iGluR4$_i$(R)-S1S2.

Data collection	Glutamate	AMPA
Space group	$P2_1$	C2
Cell parameters (Å, °)	47.4, 105.2, 66.5 $\beta = 97.2$	105.2, 169.5, 73.9 $\beta = 120.6$
Wavelength (Å)	1.0412	1.0412
Resolution limits (Å)	27.75–1.40(1.48–1.40)[a]	33.17–1.90(2.00–1.90)
Completeness (%)	98.1(100.0)	99.8(100.0)
Redundancy	3.6(3.2)	3.7(3.7)
R_{merge} (%)	9.2(40.5)	7.5(37.6)
$I/\sigma I$	11.3(2.3)	12.4(3.7)
Reflections used/R_{free}	123 064/1250(1%)	87 195/4371(5%)
Refinement		
Bond length mean deviation (Å)	0.009	0.005
Bond angle mean deviation (°)	1.23	0.88
R_{work} (%)	16.7	17.5
R_{free} (%)	18.3	21.6

[a]Values between parentheses refer to the last resolution shell.

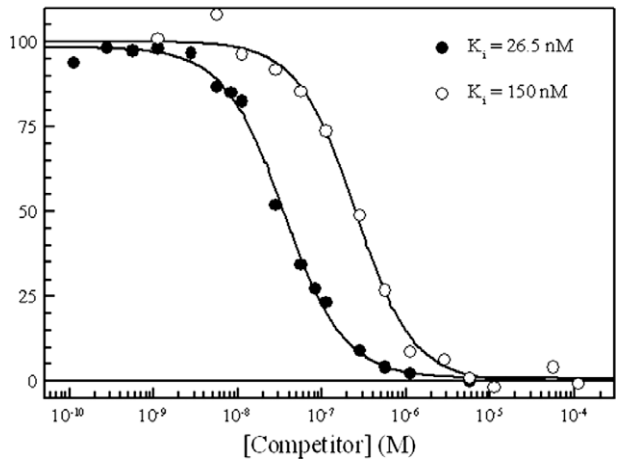

Fig. 1. Competition binding of (*S*)-glutamate and (*RS*)-AMPA at iGluR4$_i$(R)-S1S2. Shown is one competition experiment (conducted in triplicate, replicated 3–4 times) for each ligand. ○, (*S*)-glutamate; ●, (*RS*)-AMPA.

that observed for the ligand-binding core of iGluR2 [5,22]. This similarity also applies to the individual residues comprising the dimer interface and to inter-domain contacts.

The R/G editing site is located at the top of the dimer interface (Fig. 2A), and the conformation and interactions of the Arg765 side chain in iGluR4 with Arg765 from the other subunit and surrounding acidic residues are as seen in the ligand-binding core construct of the R/G unedited flip splice variant of iGluR2 (iGluR2$_i$(R)-S1S2J) [22]. In brief, the two Arg765 side chains are pointing towards each other (Fig. 2A) with counter-charges coming from Glu508 and Asp512. In the structure of iGluR4$_i$(R)-S1S2 with glutamate, a sulfate ion is located in vicinity of Arg765 and forms a salt bridge to the Nη2 atom of Arg765. Also, the flip splice variant region in the C-terminal part of the S2 segment (Fig. 2) are very similar, with water-mediated hydrogen bonds only being formed from Ser776 to the neighbouring subunit.

3.3. Structural differences among AMPA receptors

An alignment of amino acid residues comprising the ligand-binding cores of iGluR1-iGluR4 is shown in Fig. 2B. It is seen that 48 amino acids differ among all four subunits of which 20 vary between iGluR4 and iGluR2. By comparing the structures of iGluR4 and iGluR2, it emerges that these residues are primarily either facing solvent or taking part in formation of hydrophobic cores within both domains D1 and D2. Of note, five residues are pair-wise conserved between iGluR3 and iGluR4 on the one hand and iGluR1 and iGluR2 on the other hand, *i.e.* Y689F, T705V, F722Y, F724Y and V756I (Fig. 2A and B). Tyr689 in iGluR4 is located ca. 15 Å from the glutamate binding site and the side chain is engaged in water-mediated hydrogen bonds only. Thr705 is forming a direct hydrogen bond to the carbonyl oxygen atom of Thr707, which may stabilize this region harbouring the D1–D2 inter-domain lock residue Thr708 (Fig. 3C). Val756 is pointing towards a hydrophobic core in domain D1. The most important differences seem to be at positions 722 and 724. Phe724 in iGluR4 is located ca. 6 Å from the isoxazole ring system of AMPA and ca. 4 Å from the phenyl group of Phe722. Both residues are tyrosine in iGluR2, which allow the formation of water-mediated hydrogen bonds from the ligands to the corresponding Tyr723 in iGluR2 but not in iGluR4 (see Section 3.4).

3.4. Binding modes in iGluR4$_i$(R)-S1S2

The binding modes of glutamate and AMPA in iGluR4$_i$(R)-S1S2 are similar to those in the ligand-binding core of the R/G edited flop splice variant of iGluR2 (iGluR2$_o$(G)-S1S2J) [5]. Whereas the hydrogen-bonding pattern from glutamate to iGluR4 (Fig. 3A) is almost the same as in iGluR2, differences are observed in the ligand-binding sites with AMPA bound (Fig. 3B and C). The water molecule bridging Tyr723 and the ligand in iGluR2$_o$(G)-S1S2J is absent in the structure of iGluR4$_i$(R)-S1S2 with AMPA. This results in local conformational changes in iGluR4 compared to iGluR2, involving primarily residues Leu672 and Thr708. The side chain of Leu672 points towards Phe724 creating a distance of ca. 4 Å between the two residues. In addition, the inter-domain lock residue Thr708 adopts a different conformation to optimize contacts with Leu672, Phe724 and Met730. In the structure of iGluR4$_i$(R)-S1S2 with glutamate this water molecule is still present but does not form a direct hydrogen bond to the ligand. Finally, Met730 undergoes an induced fit in iGluR4$_i$(R)-S1S2 (not shown), depending on the ligand bound as previously observed in iGluR2$_o$(G)-S1S2J [23].

As no structure is yet available of the *apo* form of iGluR4$_i$(R)-S1S2, D1–D2 domain closures have been calculated relative to the *apo* structure of iGluR2$_o$(G)-S1S2J (pdb-code 1FTO, molA). Glutamate induces a domain closure of 20.6–20.8° in iGluR4$_i$(R)-S1S2, which is similar to the domain closures seen in iGluR2$_o$(G)-S1S2J (pdb-code 1FTJ: 19.1–21.1°) and iGluR2$_i$(R)-S1S2J (pdb-code 2UXA: 18.4–21.0°). Of note, AMPA introduces a larger domain closure in iGluR4$_i$(R)-S1S2 (22.9–23.5°) than in iGluR2$_o$(G)-S1S2J (pdb-code 1FTM: 21.1–21.4°), which leads to a considerably larger distance between residues in the vicinity of the Gly-Thr S1–S2 linker region from two neighbouring iGluR4 molecules (40 Å *versus* 36 Å between Cα atoms of two Ile655 residues). The location of the linkers is indicated in Fig. 2A.

4. Discussion

AMPA receptors display an overall sequence identity of 82% within the ligand-binding cores (Fig. 2B). However, the amino acid differences allow fine tuning of receptor function and also provide opportunities to specifically target a given subunit with agonists and antagonists. The structures of the ligand-binding core of the R/G unedited form of the flip splice variant of iGluR4 presented here may stimulate structure-based drug design of compounds that target specific AMPA receptor subtypes.

Compounds have been reported that show selectivity towards iGluR1/iGluR2 compared to iGluR3/iGluR4, *e.g.* (*S*)-α-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid (Br-HIBO): K_i = 250 nM (iGluR2(*R*)$_o$) and 11 700 nM (iGluR4$_o$) [11], (*S*)-α-amino-4-chloro-3-hydroxy-5-isoxazolepropionic acid (Cl-HIBO): K_i = 370 nM (iGluR2(*R*)$_o$) and 11,900 nM (iGluR4$_o$) [11] and (*S*)-2-amino-3-(1,3,5,6,7-pentahydro-2,4-dioxocyclopenta[*e*] pyrimidin-1-yl)propanoic acid (CPW399): K_i = 223 nM (iGluR2(*R*)$_o$) and 2,090 nM

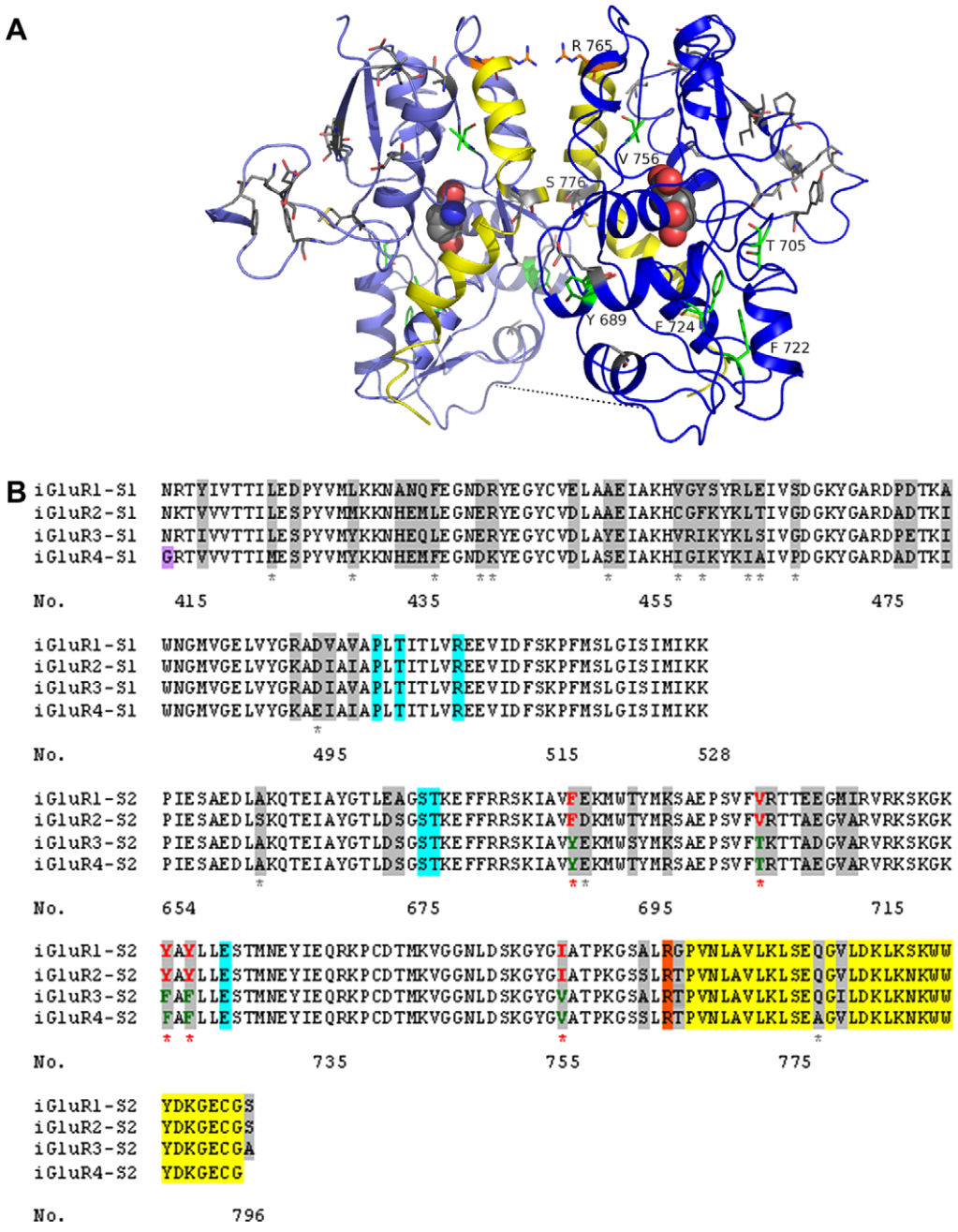

Fig. 2. The ligand-binding core of iGluR4 forms a dimer. (A) Schematic drawing of the dimeric structure of iGluR4$_i$-S1S2 in complex with glutamate. MolA is shown in light blue and molB in blue. Glutamate is shown in space-filling representation. Amino acid residues differing between iGluR4 and iGluR2 are shown in grey stick representation. The five residues in iGluR4 differing between the two AMPA receptor subgroups iGluR1/iGluR2 and iGluR3/iGluR4 are shown in green. The R/G editing site is high-lighted in orange and the flip splice variant region in yellow. The linker–linker distance between two Ile655 residues is shown by a dotted line. (B) Alignment of S1 and S2 segments of R/G unedited flip splice variants of iGluR1–iGluR4. Positions of amino acid differences are high-lighted using grey boxes and residues differing between iGluR4 and iGluR2 for which structures are available are shown by a grey star. Amino acid residues of iGluR4 forming direct hydrogen bonds to glutamate and AMPA are marked by a blue box. Protein residues that might be of importance for iGluR1/iGluR2 *versus* iGluR3/iGluR4 selectivity are marked by a red star and the residues have been coloured red and green, respectively. The R/G editing site is high-lighted by an orange box and the splice variant region by yellow boxes. The extra glycine at the N-terminal end of iGluR4 is marked with a purple box.

(iGluR4$_o$) [24]. From analyzing the structures of iGluR4$_i$(R)-S1S2 it is most likely that the iGluR4 selectivity can be attributed to the different type of amino acid present at position 724; *i.e.* a tyrosine in iGluR1/iGluR2 and a phenylalanine in iGluR3/iGluR4. When comparing the structures of iGluR4$_i$ (R)-S1S2 with AMPA and glutamate, one major difference is the absence of a water molecule in the vicinity of Phe724 in the structure with AMPA. In iGluR2$_o$(G)-S1S2J, this water molecule is present in both the complexes with glutamate and AMPA [5]. Displacement of this water molecule does not seem to have a significant effect on the binding affinity of AMPA for iGluR4$_o$ (K_i = 40 nM [11]) as compared to that at

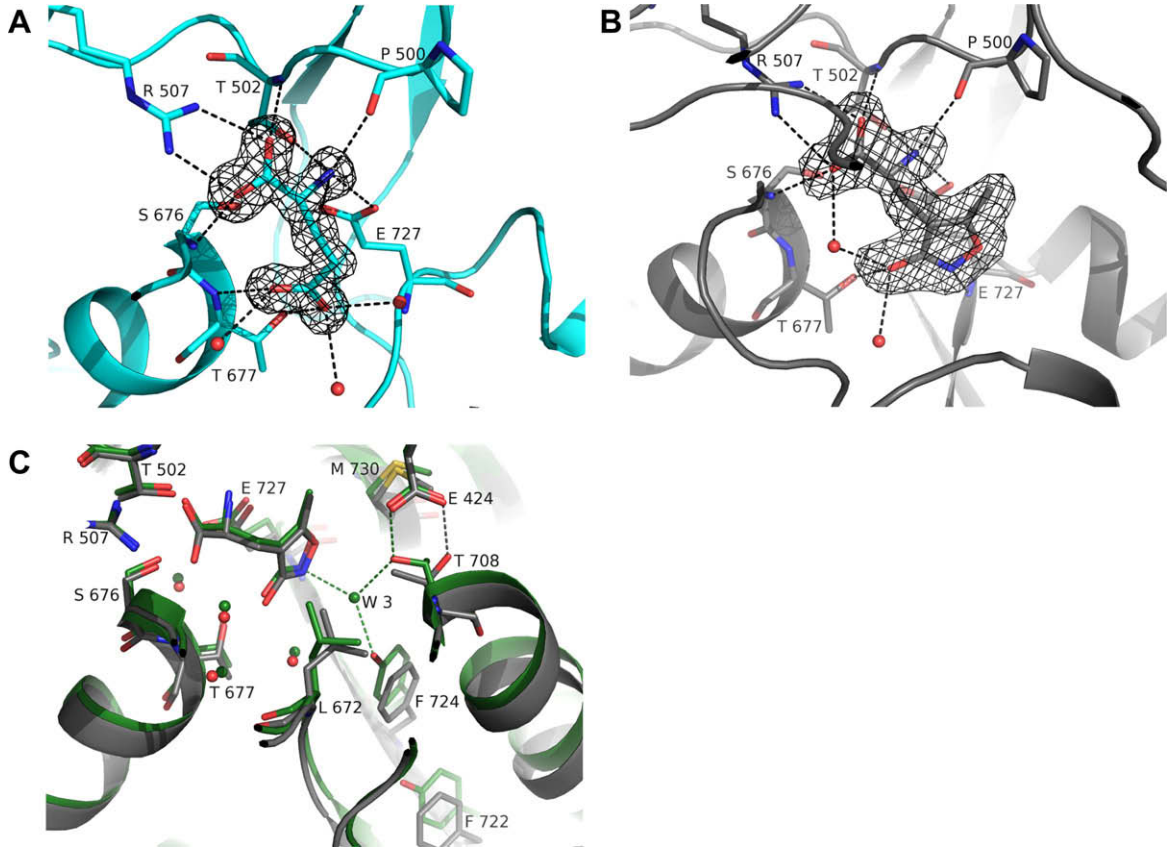

Fig. 3. Binding of agonists to the ligand-binding core of iGluR4. (A) Fo-Fc omit electron density map contoured at 3 sigma and hydrogen-bonding interactions (stippled lines) of glutamate to iGluR4 (molA) and water molecules (displayed as red spheres). (B) As in panel A, but with AMPA as ligand. (C) Comparison of the binding mode of AMPA in iGluR4 (grey; molA) and iGluR2 (green; pdb-code 1FTM, molA). The water-mediated hydrogen-bonding network from AMPA to Tyr723 in iGluR2 is shown as dotted lines; this network is missing in iGluR4 as the corresponding residue is Phe724. In addition, the D1–D2 inter-domain lock Glu424-Thr708 is high-lighted.

iGluR2(R)$_o$ (K_i = 17 nM [11]) as well as on the constructs (iGluR4$_i$(R)-S1S2: K_i = 23 nM and iGluR2$_o$(G)-S1S2J: K_i = 13 nM) [25]. Therefore, the presence of the water molecule presumably is not critical for AMPA binding. However, it has previously been shown that the selectivity of Br-HIBO and CPW399 can be attributed to the presence of this water molecule, forming a hydrogen-bonding network from the ligands to Tyr723 in iGluR2$_o$(G)-S1S2J [25,26]. This water network was shown to be absent in the Tyr723Phe mutant structures of iGluR2$_o$ (G)-S1S2J with the same two ligands, and it was proposed as an explanation for the difference in binding affinity observed at iGluR2$_o$(G)-S1S2J (Br-HIBO: 500 nM [25] and CPW399: 393 nM [26]) and the mutant (Br-HIBO: 9200 nM [25] and CPW399: 3330 nM [26]). Hence, like in the iGluR2 mutant the absence of a water molecule in the vicinity of Phe724 may be critical for binding of ligands in the binding core of iGluR4 and may explain the difference in selectivity of Br-HIBO, Cl-HIBO and CPW399. However, additional experiments are required to fully clarify the mechanism underlying the selectivity of iGluR2 over iGluR4 preferring ligands.

The dimer interface, including the R/G editing site and flip/flop splice variant region, is strikingly similar in the ligand-binding cores of iGluR2 and iGluR4. This similarity may hamper development of allosteric modulators that selectively target the dimer interface of individual AMPA receptors. The presence of similar dimer interfaces is also in agreement with the observation that the desensitization properties of AMPA receptors are alike [27], as rearrangements of the dimer interface are thought to lead to receptor desensitization [28,29]. Interestingly, AMPA induces a ca. 2° larger D1–D2 domain closure in iGluR4$_i$(R)-S1S2 than in iGluR2$_o$(G)-S1S2J. This leads to a ca. 4 Å larger separation between the linker region of two subunits (connecting both the S1 and S2 segments of the ligand-binding core to the transmembrane part). However, further structural and functional studies are required to fully establish the significance of this observation.

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